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14. ABSTRACT

Extrinsic modifications to the DNA bases such as pyrimidine dimers can arise from a variety of exposures and can lead to aberrant cell growth or death. A detailed view of this base modification is necessary for a more complete view of genetic and epigenetic regulation but the process is poorly understood. We are developing a method to look at the precise genomic position of these modifications using a next generation sequencing approach. During this research period we have been performing preliminary experiments to aid in streamlining the processes that will be used in this research. We have purified or obtained the enzymes needed for aim 1 of this research. Using these enzymes we have shown that we obtain cleavage patterns consistent with the administered UV dosage and that sequencing libraries generated for both yeast and human cells show pyrimidine bias on the 5' end, indicating that we are sequencing the dimers. Understanding where these modifications occur is a critical first step to understanding the mutations they cause.

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UV DNA modification, HeLa cells, Skin Cancer

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Introduction

Ultraviolet (UV) light damages skin cells by causing the formation of dimers on adjacent pyrimidines in DNA. These nucleobase dimers prevent proper replication and transcription, and can lead to mutation if they are not properly repaired. This project proposes to address the genomewide location of DNA modifications in human epithelial cells caused by UV light. We will determine the genomic location of nucleobase dimers created by UV light using enzymatic digestion. We will also determine the locations of mutations that are caused by UV light in cells and correlate them to modification frequencies. Understanding the initial chemical changes caused to the DNA by UV light as well as which of these modifications lead to mutation on a genomewide scale will give us valuable insight into the incidence of cancers caused by UV exposure.

Body

The Initial task tackled in the statement of work dealt with the generation of sequencing libraries in yeast and human cells. In previous work we have shown that libraries can be generated from yeast irradiated with UVC light using a commercial glycosylase and photolyases from a collaborating lab. During the process of transitioning to UVB light and human cell experiments the commercial glycosylase went off the market so the first step in this task was to generate these enzymes within the lab. UVDE is S. pombe glycosylase that can digest both CPD and 6-4 dimers. We obtained a yeast plasmid construct containing S. pombe UVDEΔ288-GST under the cup-1 promoter. We transformed this construct into yeast that are efficient for protein expression and induced them with .5mM CuSO4 for 2-4 hours. The cells were harvested, frozen and lysed by milling 3 cycles for 3 minutes. The lysate was centrifuged at 15000 rpm and the supernatant was subsequently run over glutathione column and eluted with 50mM Tris 10mM glutathione pH 8.0 over 15-1ml fractions (Fig1a) (1). This protein was concentrated and compared to the commercial UVDE enzyme from Trevigen Inc. It was determined that the homemade enzyme worked equivalently to the commercial one when used as the same concentration (Fig1b).

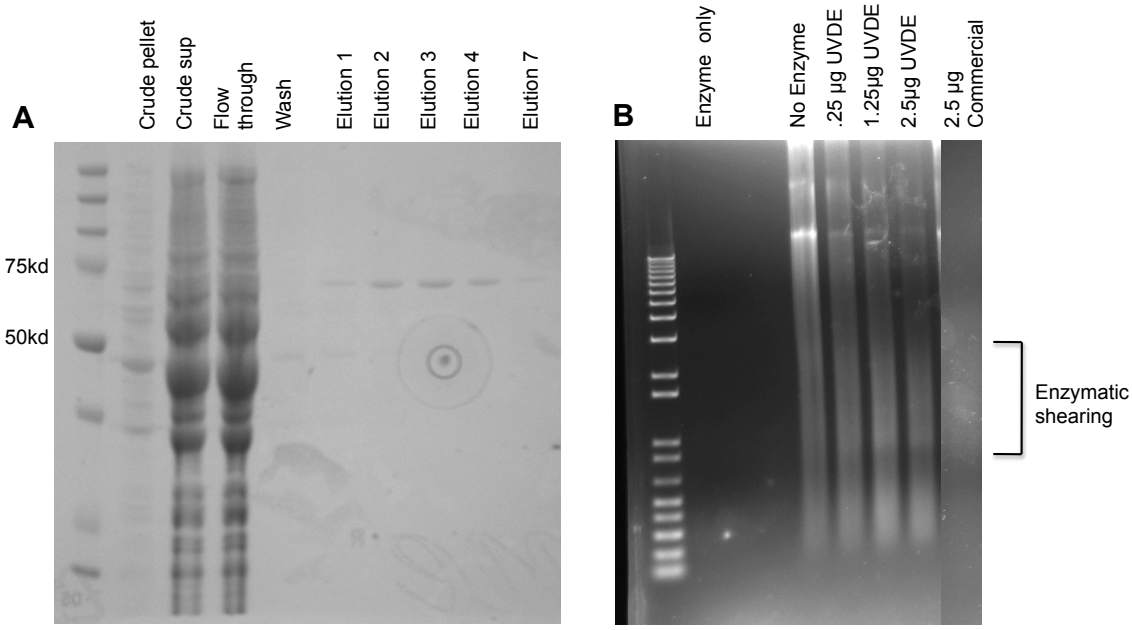

Figure 1. UVDE protein made in the lab works similarly to commercial UVDE enzyme from Trevigen. Protein was purified from yeast containing cup-1 promoter driven UVDEΔ288-GST after induction. Following purification the UVDE protein eluted in 10mM glutathione in fractions 2-4 as seen in Fig 1A. Homemade enzyme was compared to commercial enzyme for cleavage of yeast DNA treated with 10000J/m2 of UV irradiation. When used at the same concentration (compare lanes 4 &5) we obtained similar shearing patterns as outlined to the right (Fig 1b).

We next wanted to validate this enzyme for library preparation but were unable to obtain another sample of the photolyases we used for our preliminary results. We obtained constructs to make our own enzymes and went through several rounds of purification using amylose columns followed by both an S column as well as a heparin column. Although we were able to obtain relatively pure protein we were unable to validate enzyme activity through library preparation. After several tries at this we contacted a lab that purifies these enzymes for crystallization and were able to obtain a sample of both photolyases (*2, 3*).

We used our homemade UVDE as well our newly obtained photolyases and made sequencing libraries with highly irradiated yeast DNA to confirm our preliminary findings in a biological replicate. The UV treated DNA was readily sheared by our UVDE enzyme (Fig. 2A) and after treatment with either the CPD photolyase or the 6-4 photolyase, to repair the ends, sequencing libraries were obtained and run on the Illumina platform (Fig. 2b). We compared our data to a sheared control as well as the whole genome dinucleotide pattern and determined that there was a bias at the 5' end of our sample for dipyrimidines as expected if we are generating a cleavage event at damaged bases. This bias was similar if not as robust as that seen previously with the old enzymes (compare Fig. 2c to Fig. 2d).

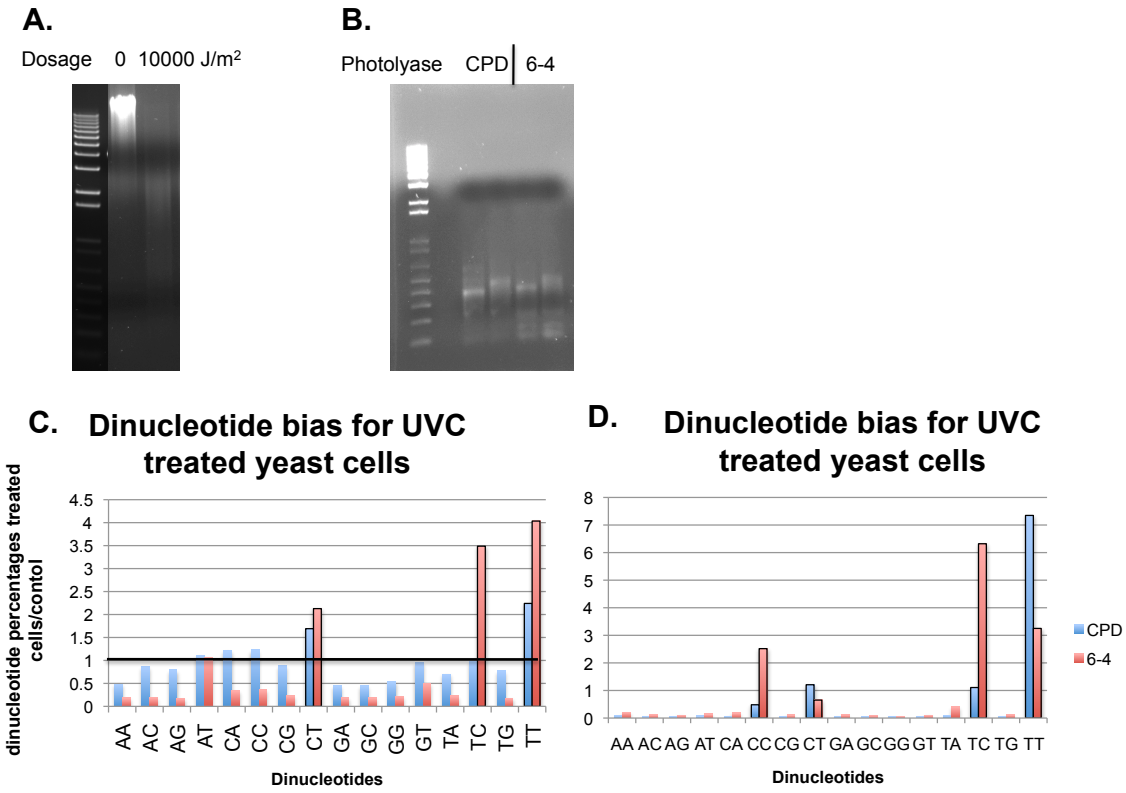

Figure 2. Illumina sequencing libraries were obtained from yeast cells treated with a high dose UVC light. Yeast cells were treated with UVC light at 0 and 10000 J/m2 and genomic DNA preps were analyzed for cleavage with UVDE (Fig. 2A). Samples were treated with CPD or 6-4 photolyase and then run through standard Illumina prep and the libraries were obtained for 2 size-selected

fractions (Fig 2B). Dinucleotide bias on the 5' end of sample reads as compared to control dinucleotide bias is shown (Fig 2C). The biased dipyrimidines are outlined in black for comparison. Dinucleotide bias from a sample prepared with commercial UVDE and photolyases from Aziz Sancar (4) is shown for comparison (Fig. 2D).

We next used our methods to map dipyrimidines in human HeLa cells. We decided to initially look at UVC to troubleshoot any problems that may occur in samples that contained more damage. We wanted to understand the lethality associated with UV irradiation to better understand which dosages would be biologically relevant for our cell types. We looked at both yeast and human cells. Yeast cells of a known density were resuspended in 5 ml of Buffer Z (1M sorbitol, 50mM Tris, 10mM BME) and irradiated at the given dose in a statalinker and the dose was confirmed using a UVP dosimeter. Cells were plated onto rich media and the irradiated samples were normalized to an unirradiated control (Fig 3A). We also looked in HeLa cells using lower doses because it has been shown that human cells cannot tolerate high doses of UV irradiation (*5, 6*). We irradiated cells in PBS and seeded fresh plates for 24 hours in DMEM before scoring with trypan blue exclusion. Counts were normalized to an unirradiated control to account for normal cell death (Fig. 3B)

A. B.

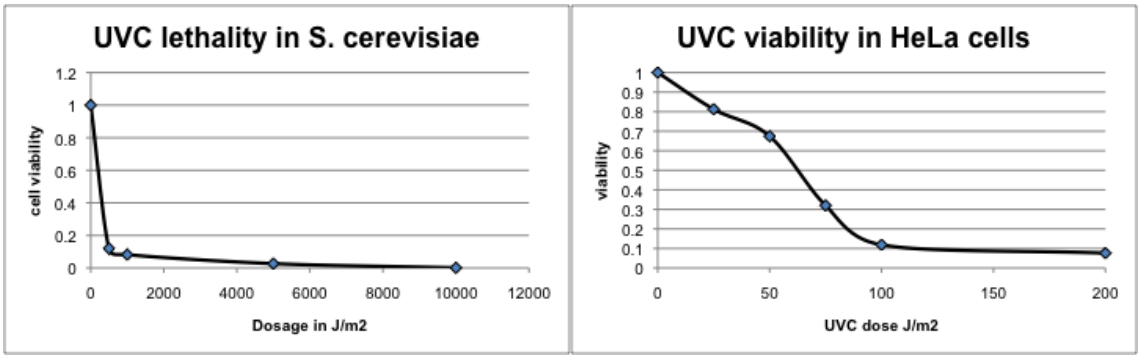

Figure 3. Human cells are 10 times more sensitive to UVC than yeast cells. Yeast cells were irradiated for the given doses and plated onto rich media at a known density. Cells were allowed to outgrow for 2 days and scored for colony formation and normalized to an unirradiated control as shown in Fig 3A. Human cells were irradiated for the given doses and plated in 6 well plates to recover for 24 hours. Cells were then trypinized and scored for viability using trypan blue exclusion and normalized to unirradiated cells as shown in Fig. 3B.

We next wanted to determine the cleavage pattern of UVDE in irradiated human cells. We grew ten 100mm plates of HeLa cells to confluency and trypsinized the cells. The cells were brought up in ½ volume of PBS and irradiated at a given dose with UVC light as measured by a spectrophotometer. The cell pellets were then collected and frozen at -80 until all samples were collected. Genomic DNA was isolated using a gentle protocol that lyses cells overnight at 55˚ in lysis buffer (100mM Tris pH 8, 5 mM EDTA, .2 % SDS, 200mM NaCl and 40µg proteinase K) followed by phenol/chloroform extraction,

DNA precipitation with isopropanol, and RNAse treatment. 2 µg of DNA for each dosage was cleaved with 1.5 µg of UVDE for 4 hours at 30˚ and ran on a gel (FIG. 4A). Cleavage to lower molecular weight fragments was seen starting at 1000J/m2 and DNA degradation was beginning to occur at 20000 J/m2. We next wanted to optimize photolyase cleavage and library preparation. We took three of our samples 0 J/m2, 500 J/m2 (low dose) and 10000J/m2 and digested 8µg with 7.5 µg of UVDE (Fig 4B). We then treated these samples with either CPD photolyase of 6-4 photolyase for 1 hour under UVA light. Samples were then column purified and run through standard Illumina preparation including polishing, a-tailing, adapter ligation and PCR. Libraries were obtained for both the low dose and the high dose samples but the low dose samples were in low abundance (Fig. 4C).

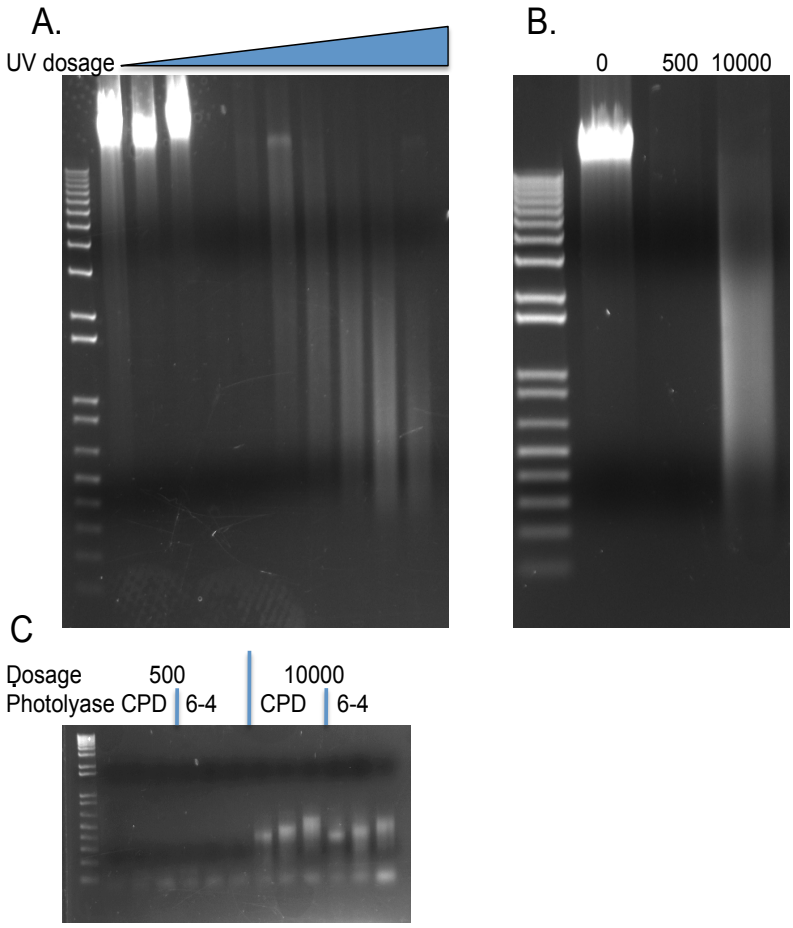

Figure 4. Illumina sequencing libraries were obtained from HeLa cells treated with low and high dose UVC light. Hela cells were treated with UVC light in increasing doses from 0 to 20000 J/m2 and genomic DNA preps were analyzed for cleavage with UVDE (Fig. 4A). Samples from 3 dosages 0, 500, and 10000 were scaled up (Fig. 4B) and treated with CPD or 6-4 photolyase. Cells were then run through standard Illumina prep and the libraries were obtained for several size-selected fractions (Fig 4C).

Libraries were pooled and sequenced on the Illumina MiSeq platform. We obtained approximately 12.5 million combined reads. For the low dose libraries only 26% of the CPD and 8% of the 6-4 libraries aligned to the hg18 build of the human genome (Table 1). This is generally a sign of low library quality and is not surprising due to the weak shearing and pcr bands. These libraries also showed no bias for dinucleotides indicating that they are not adequate UV libraries (Data not shown). The high dose UV libraries aligned much better at 71% for CPD and 73% for 6-4 libraries which is typical for human libraries (Table 1) (7). These reads were then processed to determine the dinucleotide composition on the 5' end by separating the Watson and Crick strands, and the dinucleotide percentages on the 5' end of the reads were determined for several registers around the start. The percentage of each dinucleotide combination for the whole human genome was then determined and used as a control for base bias in the genome. The data was then plotted as the percentage of each dinucleotide ratio in UV irradiated DNA/ dinucleotide ratio of the control sample. Dinculeotide bias was found in the +1 register (1st base of the read and the base previous to it) as expected considering that one base of the dinucleotide was cleaved during the photolyase repair step (Fig 5B) (8). Data from irradiated yeast treated with the same enzymes and protocols is shown for comparison (Fig 5A). Although the bias is significantly reduced compared to the yeast sample, it is present and we can continue to streamline the approach to improve our method.

Table 1.

Sample	Number of reads	Number of reads aligned	Percentage
CPD 500 J/m2	2,314,857	611653	26
6-4 500 J/m2	2,083,789	165983	8
CPD 10000 J/m2	4,573,678	3,266,064	71
6-4 10000 J/m2	3,571,113	2,601,862	73

Table 1. Data output from the Miseq sample aligned with the Bowtie alignment program.

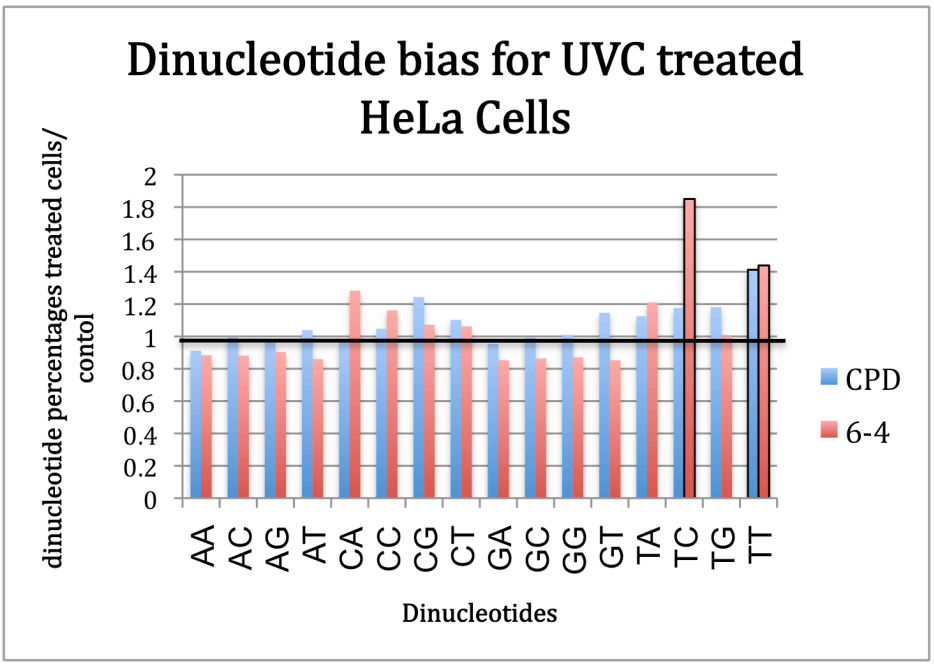

Figure 5. Sequences obtained from HeLa cells treated with UVC show pyrimidine bias on the 5' end. Samples from HeLa cells were treated with 10000 J/m2 of UVC light, sheared with UVDE, repaired with either CPD or 6-4 photolyases, and made into Illumina libraries. Dinucleotide bias on the 5' end of sample reads as compared to control dinucleotide bias is shown. The biased dipyrimidines are outlined in black for comparison.

Now that we have the appropriate enzymes and have libraries showing some bias the protocol needs to be streamlined to use UVB light at more biologically relevant dosages. To this end we obtained a UVB light from Coleman and began performing experiments but upon measuring the UV wavelength with a dosimeter determined that the UV spectrum was quite broad and all three wavelengths of UV light were being administered. To address this issue we are currently looking into an LED UVB light source that will allow us to deliver a narrow wavelength of light and will prevent contamination from other wavelengths. Once this system is obtained we will repeat these findings using UVB light. To address the issues of lowering the dosage needed to obtain a library there are several approaches that can be utilized to improve the yield of the fragments obtained. First we can try to improve shearing by UVDE with increased concentration or by increasing the incubation time. Second, we can try to repair ends more efficiently by again increasing the photolyase concentration or cleavage time, or potentially trying a different protein preparation as these enzymes have less activity than previous ones we have used. Finally we can take advantage of alternative approaches for library preparation that will allow us to mildly shear the DNA and add one adapter that is not competent for pcr unless it is paired with a second adaptor that is added following UVDE cleavage. This would allow us to get the small fragment sizes needed for Illumina sequencing without needing the large UV dosage that is required to damage the DNA every 200-800 basepairs. Using a combination of these approaches we hope to be

able to map the position of pyrimidine dimers across the human genome using a low dose of UVB light.

Key research accomplishments:

- Generation of active UVDE enzyme
- Procurement and testing of new photolyase enzyme
- Biological replicate of high dose yeast libraries with new enzymes that were similar to previous results
- Generation of lethality curves for both yeast and human cells
- Cleavage of HeLa cells with UVDE that is dependent on UV dosage
- Generation of sequencing libraries from highly dosed HeLa cells
- Validation of HeLa libraries with dipyrimidine bias on the 5' end of reads

Reportable outcomes

- manuscripts, abstracts, presentations; none
- licenses applied for and/or issued; none
- degrees obtained that are supported by this award; none
- development of cell lines, tissue or serum repositories; none
- infomatics such as databases and animal models, etc.; none
- funding applied for based on work supported by this award; none

. employment or research opportunities applied for and/or received based on experience/training supported by this award; none

Conclusion

We have obtained the enzymes needed to perform future experiments to determine the locations of UV modifications genomewide. We were able to validate these enzymes as well as perform a biological replicate of our preliminary yeast data. This was important to confirm that this method is specific for UV dimers and that the initial results we obtained were not artifactual. Although we see less bias with new enzymes the trends are consistent and we can work to further streamline the process to give us cleaner results.

We were also able to begin to make the transition into human cells. We were able to perform some initial experiments using UV on HeLa cells and showed that they were damaged as indicated by UV dimer cleavage. We were also able to make sequencing libraries from this DNA and showed that there was bias in this data for pyrimidines although there needs to be significant improvement on this protocol to be able to map the positions of UV modification genomewide. In the future we will be trying to improve our libraries by optimizing each step for the highest yield of usable sequences. Things we will try to vary include changing the amounts and incubation times of enzymes, as well as, generating a modified mapping scheme that will allow us to obtain additional small molecular weight fragments so the DNA damage needed is not so extensive. We hope that with these modifications we will be able to obtain a high level of dipyrimidine bias and we will be able to go on and accurately map the localization of UV dimers in the genome.

We have generated a method to study DNA modifications caused by exposure to UV light, which leads to two main types of DNA damage: cyclobutane pyrimidine dimers and 6-4 photoproducts. We have shown that libraries generated following high doses of UV light are capable of discerning sites of dipyrimidine modification throughout the genome, and can robustly distinguish between dipyrimidines joined by cyclobutane and 6-4 linkages in yeast. These new methods for studying genomewide distribution of UV modification may bring clarity to the relationship between UV-associated DNA modification and mutation. These data will enable us to distinguish between two models of mutation formation. The first model predicts that damage is randomly distributed throughout the genome, but DNA repair is inefficient in some regions, leading to mutations. The second model predicts that modification occurs more frequently in certain regions, and directly leads to high mutation rates in those regions. The acquisition of both modification and mutation frequencies will allow us to discern which model of mutation predominates in the human genome. This experimental approach suggests a powerful paradigm to determine how DNA modification caused by a number of other environmental exposures leads to mutation on a genome wide scale. We hope that with this new knowledge will come advancements in prevention and treatment of the skin cancers caused by these modifications.

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